IMMUNITY TO ANTHRAX: PROTECTIVE ANTIGEN PRESENT IN CELL-FREE CULTURE FILTRATES.

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EXPERIMENTS recorded in this paper show that a highly effective immunizing antigen is produced extracellularly in cultures of *B. anthracis* under certain conditions. The antigen has very similar properties to those of anthrax oedema fluid, which has been studied recently by Grabar and Staub in France and Cromartie and his colleagues in America. Although Bail's (1904) original observation on the immunity produced by oedema fluid has been confirmed many times (Okuda, 1923; Matsumoto, 1924; Hruska, 1926; Urbain and Rossi, 1926, 1927; Stamatin and Stamatin, 1936; Iványovics, 1938, and others), it is only recently that the nature of the active component has been investigated.

Staub and Grabar (1944) Grabar and Staub (1944) showed that oedema fluid contained two polysaccharides *a* and *b* both free and united with protein; *b* was immunologically identical with that found by Iványovics (1939) in the organism. Since both were precipitated by the euglobulin fraction of immune serum, which did not contain the protective antibodies (Grabar and Staub, 1942), they at first considered that they were unconnected with the protective antigen. Later, however (Grabar and Staub, 1946), they successfully immunized guinea-pigs with that fraction of oedema fluid which contained polysaccharide *a*. The activity of this fraction was removed by precipitation with the euglobulin of immune serum or by the euglobulin fraction previously absorbed with Iványovics' polysaccharide *b*. The isolated polysaccharides were inactive. They concluded that the active fraction was probably a combination of polysaccharide *a* and a protein.

Cromartie, Watson, Bloom and Heckly (1946) prepared oedema fluid in rabbits capable of protecting rabbits against 10,000 A.L.D. of spores with three weekly doses of 0·5–1·0 ml. The crude fluid was toxic, and gave rise to well-marked skin lesions in rabbits when injected intracutaneously. The toxic factor, however, was distinct from the immunizing agent and could be removed by adsorption on calcium phosphate (Watson, Cromartie, Bloom, Kegeles and Heckly, 1946). Chemical and physical investigation of the protective antigen after removal of the toxic factor showed that it was destroyed at 57° C. for 30 minutes and by trypsin and was probably protein in nature. Attempts to concentrate it by chemical means were not very successful. There was evidence that the bulk was present in the protein precipitate from 25 and 40 per cent ethanol. Electrophoresis experiments showed that it migrated between the β and γ globulins. There was not, however, a well-marked boundary, activity being present over a rather wide zone. The crude oedema fluid was also used to immunize other

animals. It was effective in sheep against 100 A.L.D., although not completely so. Partial protection was also obtained in guinea-pigs against 10 A.L.D. Doubtful results were obtained in mice. This is in agreement with most other workers (Ivánovics, 1938).

The question arose as to whether this antigen could be produced *in vitro*. Numerous attempts have been made to produce active culture filtrates with indifferent success (Sobernheim, 1913). In the light of results recorded below, two papers are worthy of mention. Casagrandi (1900) claimed to have produced some immunity in rabbits, with 30-day culture filtrates containing 1 per cent alkaline egg albumen. Later (1902) he used 50 per cent plasma cultures in broth. Large doses were given and the immunity was not of a high order. More recently Schilling (1927) used culture filtrates containing 10–100 per cent bovine or horse serum. He grew the cultures for 2–14 days and tested the filtrates in doses of 2–10 ml. in rabbits and obtained on the average 50 per cent survival. Unfortunately, the strain he used for challenge barely killed his controls in 8 days with 1 ml. broth culture. He himself concludes that no high degree of resistance was obtained. His results, however, are of some significance, since filtrates of cultures not containing serum were completely inactive. On the other hand, Basset (1933) claimed as good a result with peptone broth cultures without serum.

Recently White (1946*b*) has obtained some immunity in guinea-pigs using a non-sporing variant of the " Vollum " strain grown in whole blood, the whole culture killed with toluene being used as the vaccine.

The present paper gives the conditions for producing cell-free antigen in culture filtrates quite as active as that in oedema fluid.

TECHNICAL DETAILS.

Strains of B. anthracis *used.*

These were as follows: (1) " Vollum." This was a highly virulent strain obtained from Dr. Vollum of Oxford. The approximate A.L.D. was: for mice (intraperitoneal injection) 300 spores; for rabbits and guinea-pigs (intracutaneously or subcutaneously) 1,000 spores; for sheep* (subcutaneously) 800 spores, and for monkeys* (subcutaneously) 100,000 spores. (2) " M36." This was the " Vollum " strain after passage through monkeys, obtained from Dr. D. W. Henderson. The virulence for all animals was increased (Henderson, personal communication). (3) Strain " Hagan " from Dr. W. A. Hagan of Cornell University. This was also a virulent strain, but less so than " Vollum " or " M36." (4) " Weybridge " from the Ministry of Agriculture, Weybridge. This was an avirulent non-capsule forming strain used in the preparation of spore vaccines. (5) " HM." This was a mucoid variant of " Vollum " obtained from Dr. P. Bruce White of the National Institute for Medical Research, Hampstead. It was avirulent and did not form spores (White, 1946*a*). In addition, the following strains were obtained from the National Collection of Type Cultures: N.C.T.C. 109, 1711, 2620 and 5444. N.C.T.C. 1711 was Pasteur *premier vaccin* and 5444 was listed as avirulent. No details as to the virulence of the other

* With sheep and monkeys no accurate information could be obtained as to the A.L.D. owing to the few animals available. Of 2 sheep injected with 88 spores, both survived; of 3 given 886 spores, all succumbed in 5–7 days. One monkey given 10^4 spores survived, two given 10^5 died in 8 and 9 days, and one given 10^6 died in $2\frac{1}{2}$ days.

strains were available. Strains " Hagan " and " M36 " were the strains " G.D. 2 " and " C.D. 25 " of Cromartie, Bloom and Watson (1946).

Most of the work was done with the " Vollum " strain. Unless otherwise stated, this strain was used.

Spore Suspensions.

Except with the non-sporing strains N.C.T.C. 1711 and " HM " spore suspensions were used both for inoculating cultures and as a challenge dose in testing the efficacy of the vaccine. These were prepared as follows : The growth from 42-hour cultures on casein hydrolysate-yeast extract (CCY) agar (Gladstone and Fildes, 1940) was scraped off and suspended in distilled water and heated at 60° C. for 1½ hours. Viable counts were carried out and the suspensions stored in the ice-chest in concentrations of 2–3 $\times$ 10^{10} spores/ml. The same spore suspensions of " Vollum " and " Weybridge " were used throughout the work. Viability and virulence (of " Vollum " strain) were tested at intervals. No fall in either was found.

Vegetative Suspensions.

With the non-sporing strains " HM " and N.C.T.C. 1711, 24-hour CCY agar cultures were used. The growth was washed off in saline and used directly.

Capsulated suspensions of " Vollum " were also used. The method used for their preparation, which was worked out with the co-operation of Lord Stamp, made use of the fact that capsules are formed when *B. anthracis* is grown in an atmosphere containing high concentrations of CO_2 (Nungester, 1929 ; Sterne, 1937). Cultures were grown in CCY broth in an apparatus in which the medium (2 l.) was continually aërated with 500 ml./min. of air containing 10 per cent CO_2 finely distributed by means of a glass sinter disc. In 18 hours a heavy growth of organisms showing 100 per cent capsulation was obtained. The culture was centrifuged and an approximate standardization of quantity was made, using vaccine opacity standard suspensions.

Preparation of Culture Filtrates.

Culture vessels.

For liquid media, at first these were 100 ml. Erlenmeyer flasks. Later, and for all solid media, 8-oz. " medical flats " were used laid flat. Each contained 25 ml. medium. For larger cultures 32-oz. bottles were used containing 100 ml. medium.

Inoculum.

Dilutions of the suspensions described above were made just before use in tryptic digest of meat broth, and a known number of spores or known opacity of vegetative culture in 1·0 ml. inoculated per 25 ml. medium.

Constituents of the media.

A large number of different media were investigated. These included media made with filtered extracts of rabbit organs, muscle and skin, leucocytes and red cells and oedema fluid produced by injection of turpentine. As there was no

indication of any activity in filtrates of such cultures, the details of their preparation need not be given.

Broth.—This was either the casein hydrolysate yeast extract (CCY), or tryptic digest of beef (TMB).

Serum.—Blood was collected from the jugular veins of rabbits and sheep into agar-lined centrifuge pots. The clot was allowed to contract and the clear serum centrifuged and Seitz filtered.

Plasma.—Rabbits and guinea-pigs were anaesthetized, the thorax opened and blood removed from the heart under aseptic precautions with a bulb Pasteur pipette. 73 ml. blood was mixed with 27 ml. 2·5 per cent sterile sodium citrate solution. Heparinized rabbit plasma was also used: 500 Toronto units were given intravenously and the animals bled 10 minutes after.

Sheep and horses.—730 ml. blood from the jugular vein was received into 270 ml. 2·5 per cent sodium citrate.

Human.—This was kindly supplied by Dr. A. Aubert of the Blood Transfusion Service, Emergency Medical Service, Nottingham, and Lt.-Col. Metcalfe of the Army Blood Transfusion Service, Bristol.

All plasma was centrifuged 3 times at 3000 rev./min. to remove cells, tested for sterility and normally used without Seitz filtration.

Incubation.

This was carried out at 37° C. The " medical flats " were incubated stagnant in air, the flasks either stagnant or gently rocked on a shaker.

Harvesting the vaccine.

After various periods of growth, with liquid cultures, the organisms were centrifuged and the supernatant Seitz filtered. With coagulated plasma cultures the clot was broken up, allowed to contract, centrifuged, and the serum Seitz filtered. Agar cultures (25 ml.) were extracted with 10 ml. saline after breaking up the medium.

Production of Anthrax Oedema Fluid.

This was used as a comparison in assessing the efficacy of culture filtrate vaccines. The method of preparation was similar to that used by Cromartie, Watson, Bloom and Heckly (1946). A suspension of " Vollum " spores containing 10^5 spores in 0·25 ml. TMB was inoculated intracutaneously into the shaved skin of 6 rabbits in 4 or more areas. The animals were killed when about to die. (3–5 days), the oedematous tissue cut out, frozen and coarsely minced. It was then passed through a Latapie mincer, an equal volume of saline being added during the process. The extract was centrifuged clear from gross particles and Seitz filtered.

Animal Tests.

Animals used.

Most of the work has been carried out in rabbits (cross-bred, weight 4·5–5 lb.). Guinea-pigs, mice (albino), sheep (cross-bred Cheviot/Border Leicester) and monkeys (*Macacus rhesus*) were also used.

Active immunization tests.

Route of inoculation.—Intracutaneous, subcutaneous and intravenous routes were used with rabbits. Unless otherwise stated, the route was intracutaneous into the shaved skin of the left flank. Mice, guinea-pigs, sheep and monkeys were inoculated subcutaneously.

Number of doses and time intervals.—The procedure, unless otherwise stated, was that found satisfactory by Cromartie, Watson, Bloom and Heckly (1946), in their studies on oedema fluid. Three weekly doses were used and the animals challenged 1 week after the last dose.

Challenging dose.

A suspension of " Vollum " spores was used. For rabbits the usual challenging dose was 100 A.L.D. and consisted of 10^5 spores in 0·25 ml. TMB given intracutaneously. Guinea-pigs were also given this dose. In spite of their reputed greater susceptibility compared with rabbits, this represented approximately 100 A.L.D. tested under these conditions. Mice were challenged with 10 A.L.D., i.e. 3000 spores in 0·1 ml. given intraperitoneally. Sheep were given 125 A.L.D. (10^5 spores in 0·25 ml. TMB). In all cases animals were injected on the opposite side to that in which the vaccine was given.

Passive immunization tests.

These were carried out on mice and rabbits using sera from hyperimmunized rabbits and sheep. The sera were given intravenously in stated doses. Twenty-four hours after the animals were challenged as with the active immunity tests.

In vitro *Immunological Tests.*

Precipitation.

Hyperimmune sera from rabbits and sheep and commercial anti-anthrax horse serum were tested against serial dilutions of culture filtrates. Readings were made after 1 hour at 37° and overnight in the ice-chest.

Complement fixation.

The technique was similar to that of Fildes and McIntosh (1918). The test carried out with $2\frac{1}{2}$ and 5 M.H.D. complement. Adequate controls were included.

EXPERIMENTAL.

The Use of Anthrax Oedema Fluid as a Culture Medium.

As mentioned above, oedema fluid produced by non-specific agents in rabbits, e.g. turpentine, used as a culture medium gave entirely negative results. Specific oedema fluid was investigated. On the hypothesis that the antigen was produced in the body by the multiplication of the organism locally in the inflammatory fluid, this must contain or have contained the metabolities essential for the production of the antigen. It was considered possible that the fluid was not harvested at the point where these metabolites would be exhausted. To test this, oedema fluid heated at 60° C. for $\frac{1}{2}$ hour to destroy the antigen was inoculated

with 10^6 spores of the " Vollum " strain. The culture grew heavily, and was reaped after 18 hours. Filtrates tested in rabbits were inactive. There remained the possibility that the metabolites had been destroyed by heating, and an attempt was made to discover whether an increased amount of antigen would be found in filtrates of cultures in unheated oedema fluid. Heavy growth was obtained from small inocula. 17-hour culture filtrates tested in rabbits gave an unexpected result. The fluid had completely lost its activity. Destruction by the growth of the organism was also apparent after 7 hours : three doses of 0·5 ml., which protected all of 5 rabbits before culture, only protected 2 out of 5 after. Destruction is due to a soluble substance present in culture filtrates, probably the proteinase, since fluid incubated 3 days at 37° C. with sterile filtrates from 48-hour broth cultures of anthrax had also lost its activity. Dr. H. N. Rydon found that 85 per cent. of the protein in such mixtures had been rendered non-precipitable by protein precipitants. Some loss was also found in fluid incubated for the same time in saline. This may have been due to anthrax proteinase already present in the fluid.

These observations showed that it was necessary to test filtrates at different times during growth in order not to miss antigen which may have been formed and later destroyed.

Filtrates from Rabbit Serum Cultures.

Bloom, Watson, Cromartie and Myer (1946) have shown that the organism contains substances capable of neutralizing the anthracidal substance present in rabbit serum and tissues. In order to obtain growth in rabbit serum, it was necessary to use an inoculum containing sufficient numbers of organisms to produce the necessary concentration of the neutralizing factor. At least 3×10^8 spores in 25 ml. culture were required. Filtrates in undiluted serum and in TMB or CCY broth containing 10–50 per cent serum reaped at various times between 4 and 42 hours were consistently negative when tested in rabbits.

Filtrates from Rabbit Plasma Cultures.

As with serum, owing to the anthracidal substances present, growth could only be obtained in citrated plasma if large inocula were used. It was found, however, that if the plasma were clotted, growth was obtained with an inoculum more than 1,000 times less than that allowing growth in liquid cultures. This was attributed to a " nidus effect," a small collection of organisms localized by the coagulum being able to produce a local concentration of factor sufficient to counteract the anthracidal activity of the plasma. It was, however, necessary to remove cells (leucocytes and platelets) as completely as possible, otherwise growth was poor and in some cases non-existent. Seitz filtration (after the preliminary passage through the filter of 2·5 per cent sodium citrate to remove calcium) was unsatisfactory ; it appeared to interfere with subsequent clotting. A glass sintered funnel (No. 4, porosity 5–15μ) was found satisfactory, and the plasma was filtered through this before use.

Cultures were put up as follows : 25 ml. citrated plasma in a " medical flat " was inoculated with 1·0 ml. TMB containing known numbers of spores, mixed, and 5 ml. 2·5 per cent $CaCl_2$ (autoclaved) added. The bottle was laid flat and the plasma allowed to clot. It was incubated stagnant at 37° C. Growth occurred

as small colonies distributed through the medium, but particularly numerous where the clot was in contact with the glass. Only about one-tenth of the number of spores inoculated grew, showing that some anthracidal effect was still apparent. Three types of colonies were found: (1) dense opaque, (2) like fine thistledown, (3) like glucosazone crystals. Each of these colonies gave rise to all three types when transferred to fresh medium. They were not found with sheep, horse or human plasma, where the colonies were all similar to (1). It is possible that they represented various stages in resistance to the bactericidal substances present. Microscopically, organisms from colonies (1) and (3) were well capsulated; (2) appeared to be devoid of capsules. The culture was reaped after various times of growth, the clot being broken up, allowed to contract, and the serum Seitz filtered.

In contradistinction to the results with rabbit serum, these filtrates were highly active. Complete protection in rabbits was obtained with 3 weekly doses of 1·0 ml., and filtrates could be diluted to $\frac{1}{8}$ before the activity became greatly reduced. To avoid repetition, active filtrates will be referred to as PCF prefixed with the name of the animal from which the plasma has been obtained.

Two factors appeared to be of major importance: (1) size of culture inoculum, (2) age of culture.

Size of culture inoculum.

Table I shows the effect of different sized inocula on the production of protective antigen in rabbit plasma cultures (Rabbit PCF).

With the inoculum of 10^4 spores, filtrates were as active as oedema fluid. With an inoculum of 10^8 or greater, little or no antigen was found. This probably explained the negative results of tests of cultures in rabbit citrated plasma or serum where 3×10^8 spores were inoculated. Unless otherwise stated, 10^4 spores per 25 ml. culture were used in all subsequent tests.

Age of the culture.

Table II shows the importance of the time of reaping.

It can be seen that little antigen was formed at 12 hours. At 48 hours it was destroyed. Reapings were normally made at 18 hours.

Plasma from Other Animals.

Guinea-pig, human, sheep and horse plasmas were tested. These did not contain anthracidal substances nor was the presence of cells inhibitory. It was unnecessary to filter such plasma through sintered glass. The first sample of human plasma tested gave very poor results. When evidence as to the importance of pH was obtained (Table VI), it was found that different batches of human plasma as received from the blood depots varied considerably in pH. Some batches were as low as the critical level 6·5. When this was controlled, much more active filtrates were obtained. Table III shows the results with filtrates from different animal plasmas.

All plasmas showed activity. Subsequent work was mainly confined to sheep plasma.

TABLE I.—*The Effect of Different Sized Inocula in Rabbit Plasma Cultures on the Production of PCF. Filtrates Harvested after* 18 *hours and Injected into Groups of* 2 *Rabbits* (3 *Weekly Doses i.c.*). *Challenge* 100 *A.L.D.*

Culture inoculum (viable spores).	Dose vaccine (ml.).*	Number of rabbits surviving/number tested.	
10^4	1·0	2/2	
	0·25	2/2	
	0·125	1/2	(9)†
10^6	1·0	2/2	
	0·25	2/2	
	0·125	0/2	(2, 4)
10^7	1·0	2/2	
	0·25	1/2	(3)
	0·125	0/2	(2, 6)
10^8	1·0	0/2	(5, 7)
	0·25	0/2	(3, 4)
	0·125	0/1	(3) 1 died during immunization.
10^9	1·0	1/2	(3)
	0·25	0/1	1 died during immunization.
	0·125	0/2	(3, 4)
Controls : No vaccine	—	0/4	(4, 4, 4, 5)
Normal rabbit serum	1·0	0/2	(2, 3)
Oedema fluid	0·5	4/4	
	0·25	3/4	(14)
	0·125	2/3	(4) 1 died during immunization.

* In this and all subsequent experiments, the doses of vaccine were made up to 1·0 ml. with saline where necessary before injection.

† The numbers in brackets refer to the time of death in days.

TABLE II.—*The Effect of the Age of the Culture on the Production of Rabbit PCF. Inoculum* 10^4 *Spores.* 3 *weekly doses. Challenge* 100 *A.L.D.*

Age culture at time reaping (hours).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.	
12	1·0	1/1	1 died during immunization.
	0·25	0/2	
	0·125	0/2	
18	1·0	2/2	
	0·25	2/2	
	0·125	1/2	
30	1·0	2/2	
	0·25	2/2	
	0·125	1/2	
48	1·0	0/2	
	0·25	0/2	
	0·125	0/1	1 died during immunization.
Controls : No vaccine	—	0/4	

TABLE III.—*Presence of PCF in Culture Filtrates with Plasma from Different Species. Cultures Reaped at 18 hours. Three weekly doses of Vaccine (i.c.) in Groups of Rabbits. Challenge 100 A.L.D.*

Animal from which plasma was obtained.	Culture inoculum (spores).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.
Sheep	10^3	1·0	2/2
	10^4	1·0	2/2
		0·5	2/2
		0·25	2/2
		0·125	1/2
		0·0625	1/2
	10^6	1·0	2/2
	10^9	1·0	3/4
Horse	10^3	1·0	1/2
	10^5	1·0	2/2
	10^6	1·0	0/2
Guinea-pig	10^4	1·0	2/2
Human (pH 7·0)	10^4	1·0	4/4
		0·5	6/6
		0·25	3/4
		0·125	2/6
Controls : No vaccine		—	0/4

Properties of PCF from Sheep Plasma Cultures.

Protective effect in rabbits.

A summary of the results of 13 experiments using 91 rabbits is given below, using the standard three weekly doses i.c. and challenging one week later with 100 A.L.D.

Dose vaccine (ml.).	Number of rabbits surviving/number tested.
1·0	31/31
0·5	15/17
0·25	17/20
0·125	13/18
0·0625	3/5
Controls	0/52

Degree of immunity produced in rabbits.

Animals killed 14 days after challenge with the usual 100 A.L.D. showed no evidence of harbouring the organism in the spleen or other parts of the body. Where the animal had been given sufficient vaccine to produce a solid immunity, no evidence of a local lesion at the site of challenge was found. With less immune animals the presence of a nodule which might become necrotic and ulcerate similarly to that described by Cromartie, Bloom and Watson (1946) was noted. In still less immune animals there was considerable oedema which tracked

ventrally. If the animal survived this did not resolve, but became purulent, and it was quite common to find in these partially immune animals a large cold abscess filled with thick doughy pus which, however, was completely sterile.

To determine whether rabbits would resist greater challenging doses than 100 A.L.D., groups of 2 rabbits were given the usual three weekly doses of 1·0 ml. sheep PCF and challenged with 10^6, 10^7 and 10^8 spores. These represent 1000, 10,000 and 100,000 A.L.D. respectively. All the animals survived. Post-mortem examinations were carried out in two animals challenged with 100,000 A.L.D. and killed 14 days after. From a small area of necrosis in the centre of small, very localized fibrotic lesions at the sites of injection a few organisms were cultured. No organisms were seen microscopically. Growth could be obtained from saline suspension of the necrotic tissue heated at 60° for 1½ hours. The organisms present were, therefore, spores which had not germinated. Livers, spleens, regional lymphatic glands, blood and bone marrow were completely sterile.

Number of injections required for immunity.

Table IV shows that a minimum of two weekly injections were necessary. In order to allow a safety margin, three doses were normally used. Cromartie and Julianelle (personal communications) also found a minimum of two doses necessary with oedema fluid.

TABLE IV.—*Effect of Number of Doses of Sheep PCF on Immunity Response in Rabbits. Challenge* 100 *A.L.D.*

Number weekly doses (i.c.).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.
1*	1·0	0/2
	0·5	0/2
	0·25	0/2
	0·125	0/2
2	1·0	2/2
	0·5	2/2
	0·25	2/2
	0·125	0/2
3	1·0	2/2
	0·5	2/2
	0·25	2/2
	0·125	2/2

* A further test was carried out with a single dose of 1·0 ml. in 4 animals. Two survived.

Route of inoculation.

This was found to be unimportant. No significant difference was found between intracutaneous, subcutaneous and intravenous routes.

Thermolability.

This was of the same order as the antigen in oedema fluid (Watson, Cromartie, Bloom, Kegeles and Heckly, 1946). It was completely destroyed at 60° C. in 1½ hours. Further experiments with shorter times and lower temperatures are being undertaken.

Absence of toxicity.

There was no indication of the presence of an inflammatory factor in PCF such as that described by Cromartie and his colleagues in oedema fluid. Some evidence of local sensitization with the second and third intracutaneous doses was obtained in rabbits, but this was no more marked than a control with normal sheep serum and was attributable to the foreign protein. Homologous PCF gave very slight local erythema after the second and third intracutaneous injections. When given subcutaneously no reaction was observed. Human PCF was injected subcutaneously in man in three weekly doses. No reaction, either general or local, was obtained.

It may be remarked that, in contrast to the results of the American workers, oedema fluid produced in this laboratory using the "Vollum" strain was also devoid of toxic factor.

PCF has not yet been tested for aggressive action. Cromartie (personal communication), however, was unable to confirm Bail's observations on the aggressive action of oedema fluid.

Storage tests.

Samples of sheep PCF were stored at H ion concentrations ranging from pH 4·9–8·76 at room temperature and in the ice-chest. The pH was adjusted by the addition of small quantities of HCl and NaOH. The samples were tested at intervals for their protective effect in rabbits.

When stored at room temperature at pH 7·12 for 180 days, 3 doses of 0·25 ml. still protected both of two rabbits. On either side of this pH, however, considerable loss in activity was found. At pH 8·76 no significant loss was obtained after 29 days, but complete loss (0·5 ml. doses) in 180 days. At more acid pH values the loss was more rapid. At pH 5·95 loss in activity was apparent after 29 days and was complete in 180 days. At pH 4·9 loss was evident after 9 days.

When stored in the ice-chest (+ 1° C.) no significant fall in activity was found between pH 5·95 and 8·76 in 180 days. Activity, however, at pH 4·9 showed evidence of loss in 29 days and was completely lost in 180 days. Watson, Cromartie, Bloom, Kegeles and Heckly (1946) also noted the instability of oedema fluid antigen at low pH.

Nature of the Antigen.

As the American workers found with their oedema fluid antigen, chemical fractionation of PCF was difficult. Dr. H. N. Rydon showed that it was non-dialysable and probably protein in nature. He attempted to fractionate it by both alcohol and ammonium sulphate precipitation. No clear-cut separation was obtained; moreover, there was some evidence of destruction. It was decided to investigate the possibility of simplifying the medium used in the production of PCF before continuing further attempts at fractionation (see below).

Production of PCF by Strains other than "Vollum."

Table V gives the results of tests in rabbits using sheep plasma culture filtrates of the eight other strains of *B. anthracis*. Four strains of *B. cereus* were also included, none of which, however, was capsulated.

TABLE V.—*Production of PCF by Various Strains of* B. anthracis *and* B. cereus. *18-hour Culture Filtrates in Sheep Plasma Tested in Groups of Rabbits. Three weekly doses of* 1·0 *ml. Challenge* 100 *A.L.D. "Vollum" Spores.*

Strain.	Virulence.	Spores.	Capsules.	Number of rabbits surviving/number tested.
B. anthracis :				
"Weybridge"	0	+	0	8/8
"Hagan"	+	+	+	3/3 1 non-specific death.
"M36"	+++	+	+	4/4
"HM"	0	0	+ (on agar, in air)	0/4
N.C.T.C. 109	?	+	+	0/4
1711	0	0	+	4/4
5444	0	+	+	0/4
2620	?	+	+	2/4
"Vollum" (control)	++	+	+	8/8
B. cereus. All of four strains				
N.C.T.C. 945, 2599, 2601, 6349	.	.	.	0/4

The culture inoculum of the non-sporing strains was standardized to an opacity equivalent to 10^5 spores/ml. It is not known what this represents in viable bacilli. Assuming a bacillus has the size of 4 spores and allowing for a difference in viability between spores and bacilli, the inoculum was estimated to be approximately 2×10^4 bacilli.

From Table V it will be seen that there does not appear to be any relation between virulence, sporulation or capsulation and the production of PCF. No reputedly virulent strain, however, was devoid of activity. Of the avirulent strains, two are noted to be effective living vaccines: N.C.T.C. 1711 (Pasteur *prémier vaccin*) and "Weybridge." These both produced PCF. Strain N.C.T.C. 5444 which was devoid of activity was tested as a living spore vaccine in rabbits. The doses and time intervals were, as far as possible, the same as those giving full protection with the commercial "Weybridge" vaccine, *viz.*, one dose each of 5×10^7 and $2{\cdot}5 \times 10^8$ spores at 14 days' interval in 8 rabbits. Challenge dose of 100 A.L.D. "Vollum" spores was given 14 days later. All animals died. There appears, therefore, to be a correlation between efficiency as a living vaccine and ability to produce PCF. In this connection it may be noted that oedema fluid produced by avirulent non-capsulated vaccine strains is known to contain the protective antigen (Stamatin and Stamatin, 1936; Grabar and Staub, 1946).

Test of the Presence of PCF in Organisms.

Capsulated organisms grown in broth aerated with air + CO_2.

Cultures of "Vollum" strain in CCY broth were aërated with air containing 10 per cent CO_2 as described above. In 18 hours all organisms were capsulated. They were centrifuged, the supernatant broth removed and the deposit suspended in saline. They were mechanically disintegrated by a modification of the method used by Curran and Evans (1942). Ten ml. suspensions of opacity 40 (Burroughs Wellcome vaccine opacity standard) were placed in 2-oz. screw-capped bottles containing 10 g. glass beads (Ballotini No. 13, 0·228 mm. diam.). 0·1 ml. tributyl

citrate* (anti-frothing agent) was added, the screw caps replaced, and the whole shaken for 2 hours vertically at 400 shakes/min. (amplitude 2 in.). Microscopically, the appearance was an amorphous mass staining pink and blue with methylene blue in which no organism was seen. Plating showed that the suspensions had been sterilized. Three weekly injections of 1·0 ml. subcutaneously of $\frac{1}{4}$ dilution gave no protection in 4 rabbits when challenged one week later with 100 A.L.D. "Vollum" spores. Eight other rabbits were injected with doses ranging from 0·3 ml. of a dilution $\frac{1}{4}$ to 1·2 ml. given intravenously three times a week for 6 weeks. Two were challenged 2 months after the last dose. Both succumbed. The organisms grown under these conditions do not, therefore, appear to contain the protective antigen.

Organisms grown in plasma cultures under conditions giving optimum PCF production in the culture filtrate.

The bacilli from ten 18-hour cultures in 25 ml. sheep plasma were suspended in saline, freed from plasma clot by filtration through muslin and washed twice with 200 ml. saline. They were then suspended in 10·5 ml. saline and the opacity of the suspension determined (opacity 32, Burroughs Wellcome standard). It was diluted 1/3·2 placed in 2-oz. screw-capped bottles, tributyl citrate (0·1 ml.) added and shaken for 2 hours as described above. A control was used consisting of 10 ml. PCF *plus* 0·1 ml. tributyl citrate shaken under the same conditions. The suspension was tested for sterility, and groups of 4 rabbits were given three weekly injections i.c. of 1·0 ml. of each material and challenged in the usual way. With the organisms, all four animals died, in spite of the fact that the dose in each rabbit corresponded to the number of organisms present in 22·5 ml. culture, or about 30 times the amount of filtrate sufficient to give complete protection (3 doses of 0·25 ml.). With PCF shaken for the same time under the same conditions, 3/4 survived. Some destruction of PCF due to shaking was therefore apparent, but it is unlikely that the complete absence of activity in the organisms could be accounted for in this way. It seems, therefore, that the antigen is produced extracellularly, and is not present in the organisms even under conditions where active antigen is produced.†

Protective Tests in Animals other than the Rabbit.

Guinea-pigs.

Rabbit, guinea-pig and sheep plasma culture filtrates were used in groups of 6–8 guinea-pigs in doses of 1·0 ml. injected subcutaneously once a week for three weeks. The challenge was 100 A.L.D. spores. Of 12 injected with sheep PCF, only 1 survived 14 days. There was definite evidence of prolonged survival time in the others, all of 16 controls being dead within 4 days, and 5 injected animals surviving 5–11 days. The guinea-pig PCF also gave prolonged survival time in 7 animals used (mean 5·4 days) compared with the controls (mean 3·5 days). No evidence of protection was found with rabbit PCF.

These results are in agreement with most other workers, who find that the

* This was shown to have no action on the antigen.

† An alternative suggestion has been communicated by Mlle. Staub that the antigen was destroyed by the proteinase liberated from the ruptured cells. This hypothesis is being tested by further experiment.

guinea-pig is much more difficult to immunize than the rabbit. White's results (1946*b*) suggested that, unlike the rabbit, in the guinea-pig the immunity is not sufficient to destroy the spores, and they may remain latent in the body of the guinea-pig and may germinate to give late deaths. If this is so, it might be possible to produce complete protection against a vegetative inoculum, although failing to do so with spores.

Mice.

Groups of 10 mice were injected with 0·5–1·0 ml. sheep and human PCF subcutaneously and intravenously. Three and four weekly injections were made and the mice challenged with 10 A.L.D. spores intraperitoneally. No evidence of any protection was obtained.

Sheep.

Three doses of 10 ml. sheep PCF given subcutaneously protected all of three sheep against 125 A.L.D. spores injected one week later. Three controls died in 3 and 4 days. These sheep were subsequently hyperimmunized to produce sera. One sheep which had received a further three doses of 10 ml. sheep PCF was injected with a suspension of living capsulated " Vollum " strain from an 18-hour culture, the bacterial substance being equivalent in opacity to 10^8 spores. Assuming the vegetative organism was four times the size of a spore, this would represent about 30,000 A.L.D. It survived, no reaction, general or local, being observed.

A further test with 2·0 ml. doses was carried out by Lt.-Col. J. Barnes in two sheep. Three weeks after challenge with 125 A.L.D. both animals were alive and well.

Monkeys.

Groups of 2 *Macacus rhesus* were given three weekly doses subcutaneously of 1·0 ml. sheep and human plasma culture filtrates. One week later they were challenged subcutaneously with 10^7 spores (approximately 100 A.L.D.). The controls died in 4 and 5 days. All the vaccinated monkeys survived, and no reactions were observed.

Analysis of the Culture Medium to Determine the Constituents Responsible for the Production of PCF.

Thermostability of the essential constituent.

A series of 18-hour cultures was put up with sheep plasma heated to different temperatures before inoculation. Cultures heated 1½ hours at 56° C. grew well, but clotting was inhibited. All of 7 rabbits injected with the usual three doses of 1·0 ml. survived 100 A.L.D. With plasma heated 1½ hours at 70° C., 1 out of 6 survived. The plasma was coagulated so that it was difficult to estimate the amount of growth, but films showed that growth was appreciable. Plasma steamed and autoclaved 20 min. 115° C. was completely ineffective. It would seem therefore that the essential constituent was destroyed at 70° C. for 1½ hours, the coagulation temperature of proteins.

The effect of pH on the production of PCF.

Table VI shows the effect of growth at different pH on the production of PCF in sheep plasma cultures.

TABLE VI.—*The Effect of pH on Production of PCF. Protective Effect in Rabbits Tested under the Standard Conditions. Culture: Sheep Plasma, Inoculum* 10^4 *Spores; 18 hours' growth.*

Initial pH.	Approximate estimation of amount growth (no. colonies × 10^3).	Final pH.	Dose PCF i.c. (ml.).	Number of rabbits surviving/ number tested.
8·43	2	7·6	0·5	2/2
			0·25	1/2
			0·125	1/2
			0·0625	1/2
7·66	5	7·6	0·5	1/2
			0·25	2/2
			0·125	2/2
			0·0625	1/2
6·96	10	7·0	0·5	2/2
			0·25	2/2
			0·125	1/2
			0·0625	1/2
6·5	10	6·5	0·5	0/2
			0·25	0/2
			0·125	0/2
			0·0625	0/2
6·04	5	6·2	0·5	0/2
			0·25	0/2
			0·125	0/2
			0·0625	0/2
5·6	No clot, poor growth	6·2	0·5	0/2
			0·25	0/2
			0·125	0/2
			0·0625	0/2

Table VI shows a very clear-cut result: PCF was not produced in cultures at pH 6·5 or lower. There is no significant difference between results with filtrates produced at pH 6·96–8·43. This is not due to a direct effect of pH on PCF, since it can be stored overnight at 37° C. at pH 6·0 without loss.

Fractionation of Plasma to Determine the Metabolites essential for the production of PCF.

Serum.—The experiments recorded above were all carried out using whole plasma. Tests were now made with serum.

Rabbit serum.—It has been pointed out that cultures in rabbit serum failed to grow unless large inocula were used, and that with such inocula no PCF was obtained. Growth, however, could be produced with small inocula (10^4 spores)

if the medium were made solid or semi-solid with agar (1·0–0·5 per cent). Saline extracts of such cultures (25 ml. extracted with 10 ml. saline) protected 2 of 4 rabbits tested with doses equivalent to 1 ml. undiluted serum. Heparinized plasma similarly treated gave no better protection, so that the results cannot be attributed to a difference between serum and plasma, but rather to the interference by agar of PCF production.

Sheep serum.—There are no substances inhibitory to growth in sheep serum, and undiluted serum cultures inoculated with 10^4 spores grew well. Filtrates of 18-hour cultures tested in falling doses in rabbits under the standard conditions gave the following results :

Dose vaccine (ml.).	Number of rabbits surviving/number tested.
1·0	9/10
0·5	3/5
0·25	4/5
0·125	3/5
0·0625	1/5
Control (no vaccine)	0/5

If these results are compared with the results obtained with plasma there is a slight indication that sheep serum is less effective than plasma ($\chi^2 = 6{\cdot}15$; $P = 0{\cdot}047$). For the comparison, since the doses are the same in each series, a total of all animals surviving irrespective of dose has been taken. Further experiments, however, comparing dilutions of plasma and serum in culture media, showed that there was little difference between them (Table VII) ($\chi^2 = 0{\cdot}095$).

TABLE VII.—*A Comparison between the Protective Effects in Rabbits of Filtrates of Cultures in Various Dilutions of Sheep Plasma and Serum. Standard 3 weekly doses* 1·0 *ml. i.c. Challenge* 100 *A.L.D.*

Dilutions of plasma* or serum in culture.	Number of rabbits surviving/number injected with serum culture filtrate.	Number of rabbits surviving/number injected with plasma culture filtrate.
Undiluted	9/10	4/4
1/2	3/4	4/4
1/4	5/6	5/6
1/8	3/8	5/7 (1)
1/16	3/7 (1)†	4/8
1/32	1/3 (1)	0/4
Control (filtrate from culture in 25 per cent TMB)	0/4	
Control (no vaccine)	0/4	

* In order to keep the amount of growth as constant as possible TMB was added in a final concentration of 25 per cent. It has been found that this medium gave approximately the same amount of growth in 18 hours as undiluted sheep serum. With plasma cultures, $CaCl_2$ was added in amounts sufficient to remove the citrate ion. No clotting, however, occurred at dilution of 1/8 and lower.

† Numbers in brackets refer to non-specific deaths during immunization.

The effect of dialysing serum and plasma.

Initial experiments were done with sheep plasma. Later, serum was used with similar results. With plasma the dialysis, carried out by Dr. Rydon, was as follows :

250 ml. citrated plasma dialysed against 750 ml. distilled water for 17 hours

Residue.	*Diffusate.*
Dialysed against 0·67 per cent sodium citrate in 0·9 per cent saline for 2 days with frequent changes. Volume 270 ml.	Concentrated *in vacuo* below 35° C. to 120 ml. (contains 75 per cent of diffusible material).

Serum was dialysed in the same way except that citrate was not used in the saline. Each fraction was Seitz filtered and cultures were put up as follows, using as a control Seitz filtered plasma. In order to keep the amount of growth constant, TMB was added to a concentration of 25 per cent.

(1)	Diffusate 16 ml.				+	TMB 6 ml.	+	saline 3·2 ml.				
(2)	„ 8 „				+	„	+	„ 11·2 „				
(3)			Residue 16 ml.		+	„			+	2·5% $CaCl_2$	3·2 ml.	
(4)			„ 8 „		+	„	+	„ 9·6 „	+	„	1·6 „	
(5)	Diffusate 8 „	+	„ 8 „		+	„	+	„ 1·6 „	+	„	1·6 „	
(6)	Plasma 16 „				+	„			+	„	3·2 „	
(7)	„ 8 „				+	„	+	9·6 „	+	„	1·6 „	

All cultures remained fluid, clotting in (6) and (7) being inhibited by Seitz filtration. In 18 hours little difference was noted in the amount of growth, but growth in (3) and (4) was granular. Capsule production was well marked in cultures (5), (6) and (7), but was absent in cultures (1), (2), (3) and (4). 18-hour culture filtrates were tested in rabbits using the standard conditions (Table VIII).

TABLE VIII.—*Evidence of Two Factors in Plasma Required for the Production of the Immunizing Antigen. Groups of 4 Rabbits Immunized under the Standard Conditions with Culture Filtrates in Fractions of Plasma Separated by Dialysis.*

Culture filtrate (see text).	Approx. concentration (per cent of plasma).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.
(1) Diffusate	100	1·0	0/4
(2) „	50	1·0	0/4
(3) Residue	57	1·0	0/4
(4) „	28·5	1·0	0/4
(5) „ + diffusate	50 + 28·5	2·0	4/4
(6) Whole plasma	60	1·0	4/4
(7) „ „	30	1·0	4/4

A similar result was obtained with dialysed serum.

It is clear from this result that two factors present in plasma are both necessary. One of these factors is dialysable, the other is retained. That the two factors are required in the production of the immunizing antigen and not in its protective effect is shown by pooling culture filtrates (1) and (3) (Table VIII) and testing the mixture in rabbits. No protection was obtained.

Estimation of the amount of each factor required.

Cultures were put up containing a constant amount of one factor and falling concentrations of the other. Table IX shows the results when filtrates from these cultures were tested in rabbits.

TABLE IX.—*The Optimal Concentrations of each of the Two Factors in Plasma Cultures Necessary for the Production of the Immunizing Antigen. Tests in Rabbits :* 1·0 *ml. Filtrate given i.c.* 3 *weekly doses. Challenge* 100 *A.L.D.*

Basal medium : *TMB diluted to* 25 *per cent after addition of factors. Final volume* 25·7 *ml. Inoculum* 10^4 *spores in* 0·5 *ml. TMB.* 2·5 *per cent* $CaCl_2$ *was added in the proportion of* 1·6 *ml. to* 8 *ml. dialysed plasma to remove the citrate ion.*

Diffusate } *As in Table VIII.*
Residue }

Residue.		Diffusate.		Per cent organisms showing capsules.	Number of rabbits surviving/number tested.
Volume (ml.	Per cent whole plasma.	Volume (ml.).	Per cent whole plasma.		
8	29	8	50	75	4/4
8	29	2	12·5	50	3/4
8	29	0·5	3·1	v. few	1/4
8	29	0·125	0·8	0	1/4
8	29	0	0	0	0/4
4	14·5	8	50	75	4/4
2	7·2	8	50	75	3/4
1	3·6	8	50	75	2/3 (1)
0·5	1·8	8	50	50	2/4
0	0	8	50	0	0/3 (1)
Control (no vaccine)					0/4

Similar results were obtained with serum.

Numbers in brackets refer to non-specific deaths during immunization which have been excluded from the denominator.

From these results it would seem that dialysed plasma cannot be diluted below 14·5 per cent and diffusate not below 50 per cent without loss of activity. It would also appear that under these conditions both factors are required also for capsule production. However, there is no question of capsulation playing a part, since the " Weybridge " strain produced no capsules, and yet in the presence of both factors gave active culture filtrates.

Replacement of the diffusate factor with sodium bicarbonate.

It was found that the diffusate factor could be replaced by sodium bicarbonate. A solution in saline ($M/2$; 4·2 per cent) was sterilized by filtration and added to cultures containing 29 per cent dialysed sheep plasma and 25 per cent TMB to make concentrations of 1·0, 0·2 (approximately equivalent to the concentration in plasma) and 0·04 per cent. Cultures were incubated for 18 hours after inoculation with 10^4 " Vollum " spores. With 1·0 per cent, some inhibition of growth was found, presumably due to the high pH.

Capsulation was well marked with 1·0 and 0·2 per cent, but was absent with 0·04 per cent., showing that the factor responsible for capsulation in the diffusate

could also be replaced by bicarbonate. Tests in groups of 3 rabbits under the standard conditions gave complete protection with 1·0 and 0·2 per cent. With 0·04 per cent, all 3 died. A concentration of bicarbonate equal to that in plasma, therefore, is effective, but whether the activity of the diffusate is entirely dependent on its content of bicarbonate is still under investigation. Table IX suggests that the diffusate factor is active in higher dilutions: 12·5 per cent (equivalent to 0·025 per cent sodium bicarbonate) produced filtrates which protected 3/4 rabbits, whilst 0·04 per cent sodium bicarbonate gave no protection when tested in 3 rabbits. However, with such small numbers of animals it is impossible to obtain anything like an accurate estimate. The effect of sodium bicarbonate was shown not to be due to pH.

Carbon dioxide.

In order to test whether carbon dioxide would replace the dialysate factor, cultures in 29 per cent dialysed plasma and 25 per cent TMB were incubated in equilibrium with 5 per cent CO_2 in air. The cultures were saturated with air containing this concentration of CO_2 and incubated stagnant in McIntosh and Fildes' jar filled with the gas. The pH fell to 6·5 immediately after gassing. A good capsulated growth was obtained, but filtrates were inactive. The concentration of HCO_3' in equilibrium with 5 per cent CO_2 at pH 6·5 is 0·00285 *M*. This corresponds to a concentration of $NaHCO_3$ well below that found to be effective in producing the antigen. At higher pH values the equilibrium would shift so that more and more bicarbonate would be present. It is impossible therefore to test the effect of CO_2 in the virtual absence of bicarbonate ions at pH values above the critical value for the production of the antigen, i.e. pH 6·5 (Table VI). The relation between CO_2 and capsule production and the use of CO_2 as a standard method of producing capsulated organisms has been pointed out. It seems, therefore, that for capsule production, in contrast to the production of the immunizing antigen, CO_2 and bicarbonate are interchangeable. However, it is possible that the effect of bicarbonate depends on the CO_2 with which it is in equilibrium.

The nature of the residue factor retained on dialysis.

Little work has so far been done. Preliminary tests on dialysed serum fractionated by Dr. Rydon indicated that it was associated with the albumen fraction. Further work is in progress.

Passive Immunization Tests.

Preliminary tests were carried out with sera from rabbits immunized with 3 weekly doses of 1·0 ml. of rabbit and sheep PCF. The animals were bled from the jugular vein 14 days after the last injection. As a routine the sera were heated ½ hour at 56° C., but unheated sera were also used. The sera were given intravenously as described under "Technical Details" to groups of 10 mice in doses of 0·5 to 0·05 ml. and into groups of 2 rabbits in doses of 10–1·0 ml., and the animals were challenged with 10 A.L.D. (for mice) and 100 A.L.D. (for rabbits) of spores 24 hours after. No evidence of protection in either animal was obtained, although the animals from which the sera were taken had a solid immunity.

Further tests were carried out with sera from hyperimmunized rabbits and sheep. For the preparation of the sera 5 sheep and 12 rabbits were used. They

were given 2 courses of immunization, using 1–10 ml. doses of PCF in sheep and 0·3–4·0 ml. doses in rabbits. Both homologous and heterologous PCF was used in both species. Doses were given either subcutaneously or intravenously at times ranging from 1–3 times per week with 1 week's interval between courses. Bleedings were made between courses, and after the second course 1 week after the last injection. The sera were tested in groups of 20 mice in doses of 0·05–0·5 ml. intravenously and challenged with 10 A.L.D. spores 24 hours later. No indication of protection was obtained. A total of 68 rabbits given doses ranging from 10–50 ml. of these sera intravenously were challenged with 100 A.L.D. spores. There was no protection or even significant delay in death time. In other cases rabbits received two doses of serum, one given 24 hours before and the other 24 hours after challenge, again with negative results. It would seem, therefore, that the sera had no protective value, although the animals from which the serum was taken were solidly immune.

The possibility arose that whole blood might be protective, and heparinized blood from rabbits hyperimmunized with sheep PCF was tested in rabbits in doses of 50, 20 and 10 ml. No protective effect was obtained. That the rabbits were capable of being passively protected was shown by the complete protection obtained with 5 and 10 ml. doses of " Weybridge " commercial serum. This is serum from horses immunized first with the " Weybridge " living spore vaccine, followed by a course of immunization with a virulent strain. In order to determine whether an effective serum could be produced by using a virulent strain, three sheep previously hyperimmunized with sheep PCF were given living capsulated " Vollum " vegetative organisms in three weekly doses of 1 ml. of a suspension corresponding in opacity to 3×10^8, 6×10^8 and $1{\cdot}2 \times 10^9$ spores. After 1 week they were bled and the serum pooled and tested for passive protection in rabbits (Table X).

TABLE X.—*Passive Protection Tests in Rabbits using Pooled Sera from 3 Sheep Immunized with Sheep PCF Before and After a Further Course of Immunization with Living Capsulated " Vollum " Bacilli. Serum given Intravenously. Challenge 100 A.L.D.*

Serum from animals given.	Dose serum (ml.).	Number of rabbits surviving/number injected.
(*a*) Two courses immunization with sheep PCF	10	0/4 (3, 3, 4, 6)*
	5	0/4 (3, 3, 4, 5)
	2	0/4 (3, 3, 4, 4)
	1	0/4 (3, 4, 4, 4)
(*b*) Ditto + one course with living virulent bacilli	10	3/4 (6)
	5	2/4 (5, 6)
	2	1/4 (3, 5, 5)
	1	0/4 (3, 3, 4, 5)

* Numbers in brackets = survival time in days of animals which succumbed.

From Table X it is seen that immunization with living " Vollum " organisms produced sera which contained protective antibodies for the rabbit. A similar result was found with serum from rabbits treated in the same way with living " Vollum " organisms following immunization with sheep PCF. As a control, a vaccine was prepared using a suspension of capsulated organisms of the same density broken up with glass beads until all the organisms were killed, as pre-

viously described. The serum from rabbits using this vaccine (following immunization with PCF) gave no protection in rabbits. It would seem, therefore, that the living organism is necessary for the production of protective antibodies, although whether it depends on the virulence of the organism has not yet been determined. There is evidence that it does, since we have failed to obtain protective antibodies in rabbits immunized with the " Weybridge " spore vaccine alone. It may be noted that the serum described by Watson, Cromartie, Bloom, Kegeles and Heckly (1946) as protecting 50–80 per cent of their guinea-pigs was from rabbits not only immunized with oedema fluid antigen, but which had also been challenged twice with large doses of virulent spores. Matsumoto (1924) obtained poor results with filtered oedema fluid, but further immunization with unfiltered fluid containing living organisms was much more successful.

Passive protection in mice with sera from sheep and rabbits immunized with PCF, followed by living capsulated suspensions of " Vollum " strain.

Groups of 10 mice were injected i.v. with 0·5 and 0·2 ml. of serum from rabbits given two courses of immunization with sheep PCF followed by one with living capsulated " Vollum " strain. They were challenged 24 hours after with 10 A.L.D. spores i.p. The difference in numbers of deaths between groups given this serum and other groups given normal rabbit serum was not significant ($\chi^2 = 3{\cdot}3$: $P = 0{\cdot}2$). A similar result was obtained with the " Weybridge " anti-anthrax serum, which, as stated above, gave complete protection in rabbits. Tomcsik and Ivánovics (1938*b*) showed that sera containing capsular antibody had some protective value in mice, but not in rabbits. It would seem, therefore, that sera from rabbits immunized with PCF + capsulated organisms should be protective in mice in consequence of the capsular antibody they presumably contained. Precipitation tests for capsular antibody, however, using a purified polypeptide preparation (Hanby and Rydon, 1946) were consistently negative, even when sera were taken after two further courses of immunization with capsulated organisms. It appears that the capsule on the organism is a poor antigen. We have had repeated failures in rabbits and sheep not only with this strain, but also with the living " HM " strain. Tomcsik and Ivánovics (1938*a*) and Ivánovics (1940), although they had more success, found that rabbits respond irregularly, and that differences existed between bacterial strains.

Attempts to absorb the protective antibody in immune sera.

Grabar and Staub (1942) showed that the protective antibody for guinea-pigs was in the pseudoglobulin fraction of immune serum, which, however, gave no precipitation with the capsular polypeptide or somatic polysaccharide. Antibodies against these were in the non-protective euglobulin fraction. Absorption with capsular polypeptide did not remove the protective antibodies for guinea-pigs and rabbits (Staub and Grabar, 1943). Matsumoto (1924) failed to remove the protective antibody from immune sera with killed bacilli even when " animalized " organisms were used from peritoneal exudate or when the bacilli were actually grown in the immune serum. He claimed, however, to have succeeded with filtered oedema fluid. A small precipitate was formed, but it appeared immaterial whether this was filtered off or not. On the other hand, Watson, Cromartie, Bloom, Kegeles and Heckly (1946) completely failed to absorb protective antibody with oedema fluid.

Absorption with PCF.

Two sera were used: (1) " Weybridge " commercial anti-anthrax serum, and (2) pooled sera from sheep immunized with sheep PCF followed by living "Vollum" organisms. Absorptions were carried out with sheep PCF. Equal volumes of undiluted sera and PCF, undiluted and in dilution of 1/2, 1/4 and 1/10, were mixed and allowed to stand 2 hours at room temperature and then overnight in the ice-chest. A higher temperature was avoided to prevent possible destruction of PCF by anthrax proteinase that it might have contained. A slight turbidity was noticed in the tube with the highest concentration of PCF, but no precipitate. It was therefore not centrifuged. A second absorption (or neutralization) was carried out, using a volume of PCF undiluted and diluted as before in quantities equal to the volume of the first absorption mixture, thus diluting the serum 1/4. After placing in the ice-chest overnight, the mixture was injected intravenously into groups of 6 rabbits in doses of 40 ml., equivalent to 10 ml. of unabsorbed serum. A control with unabsorbed serum diluted to an equal amount with saline but otherwise treated exactly like the absorbed sera was included. The animals were challenged with 100 A.L.D. spores 24 hours after. Using the " Weybridge " commercial serum, absorption with undiluted PCF, and PCF diluted 1/2, 1/4 and 1/10 gave survival ratios of 2/6, 5/6, 4/6, 4/6 respectively. The unabsorbed serum, however, only protected 2/6. With the sheep serum, the results with the absorbed serum were 4/6, 2/6, 2/6, 1/6, and with the unabsorbed serum, 1/6. The poor results with the controls is not readily explainable, since this dose of these sera gave 75–100 per cent survival in previous tests. In spite of the somewhat irregular results, however, there is no indication of any absorption or neutralization of the immune serum by PCF.

Absorption with living organisms.

In order to determine whether the protective property of sera was dependent on antibody to an antigen in the living organism, absorption tests were carried out with washed living suspensions of capsulated bacilli and spores of " Vollum " strain. " Weybridge " commercial anti-anthrax serum was used. The absorptions were carried out as follows:

First absorption.

(1) 50 ml. serum + 50 ml. saline.
(2) ,, + 50 ml. spore suspension (3×10^9/ml.).
(3) ,, + 50 ml. capsulated bacilli suspension (opacity equivalent to 3×10^9 /ml. spores).

These were allowed to stand in the ice-chest overnight, and then spun down and the supernatant used for a second absorption.

Second absorption.

(1) Supernatant from (1): 90 ml. + 8 ml. saline (final dilution 1/2·2).
(2) ,, (2): 90 ml. + 8 ml. spore suspension ($1{\cdot}8 \times 10^{10}$) (final dilution 1/2·2).
(3) ,, (3): 60 ml. + 40 ml. capsulated bacilli suspension (opacity equivalent to 3×10^9/ml. spores) (final dilution 1/3·3).

After standing overnight in the ice-chest the suspensions were spun down and the supernatants Seitz filtered and the equivalent of 10 ml. of undiluted serum (i.e. 22 ml. of (1) and (2) and 33 ml. of (3)) injected into groups of 2 rabbits intravenously. 24 hours after the animals were challenged with 100 A.L.D. spores intracutaneously. In groups injected with sera (1) and (3), no deaths occurred. In animals injected with Group (2) serum, 1 died out of 2 injected. This animal died on the 8th day. Controls given normal serum died in 3–4 days.

It would seem, therefore, that no significant absorption by spores or capsulated organisms had taken place.

A further absorption test is being carried out with PCF together with living bacilli and with non-capsulated bacilli.

In vitro *Immunity Tests.*

Sera from animals hyperimmunized with PCF and with PCF and living organisms and other sera containing high titres of antibody against the capsular polypeptide and a polysaccharide isolated from the organism by Dr. H. N. Rydon (personal communication) were tested against sheep and rabbit PCF. Except where a reaction was due to heterologous protein, e.g. with sheep serum immune to rabbit PCF and tested against rabbit PCF, no precipitation was obtained in dilutions of antigen 1/2–1/1024 and 1/2 serum.

Complement-fixation tests were also negative. Owing to the anti-complementary effect of protein present in PCF, tests were controlled with normal sheep and rabbit serum. Specific complement fixation was only obtained where heterologous PCF was used as the immunizing and test antigen. The normal serum control showed that this was due to the heterologous protein.

DISCUSSION.

It would seem from the evidence in this paper that the protective antigen formed in plasma culture filtrates has very similar properties, and is probably identical with that described by the American workers in anthrax oedema fluid, but unlike their material, it is not associated with any inflammatory factor. It is not, therefore, necessary to postulate any interaction between the organism and its host to explain the formation of the antigen. Neither is it necessary to regard its formation as being due to an interaction between anthracidal substances in serum or plasma and substances produced by the organism, since the antigen is formed in plasma which is devoid of such substances. On the other hand, it does not seem to be present preformed in the organism, even under conditions of good production in the medium. This suggests that it is distinct from the antigens of the cell. It is formed early in growth, and is destroyed by the proteolytic enzymes of the organism. It would appear to be, therefore, formed externally by the cell and not a product of autolysis.

Of the two factors in plasma or serum necessary for production of the antigen, one is a serum protein or associated with it, probably albumen. It is possible that the antigen is formed from normal serum albumen by coupling of an active haptene (for example the polysaccharide *a* of Grabar and Staub, 1944) produced by the organism. Preliminary experiments, which will be reported in a subsequent paper, made it clear that the non-dialysable (albumen) factor had to be present in the culture medium. If suspended in the

medium in a cellophane bag, no antigen was formed either inside or outside the bag. Attempts to produce the antigen by mixing normal serum with filtrates from broth cultures containing 0·2 per cent sodium bicarbonate were unsuccessful.

What role the second (dialysable) factor plays it is impossible at this stage to conjecture, although it has been established that it can be replaced by sodium bicarbonate. It does not act as a buffer or by altering the pH of the medium. Some evidence as to whether it is incorporated in the antigen could be sought by the use of bicarbonate containing isotopic carbon. The carbonate or bicarbonate ion appears to be necessary, since it is not replaceable by CO_2.

The apparent absence of protective antibodies in the serum of animals hyperimmunized with this antigen is in accordance with the findings of most workers using oedema fluid or spore vaccines. It raises once again the long-debated question as to whether a true cellular immunity may be produced apart from circulating antibody (Teale, 1935). It is not proposed here to enter into a discussion on this point, except to state that no protective antibodies could be demonstrated in the spleen, liver and bone marrow of immune rabbits ground up in their serum. Neither was whole blood effective.

A further mystery is the nature of the protective antibodies that are produced on further immunization with virulent organisms. The complete failure to obtain any *in vitro* tests, and the failure to absorb or neutralize the antibodies with active culture filtrates or by living organisms, suggests that a still further antigen is involved which is produced by virulent cells only in the body. Yet Watson, Cromartie, Bloom, Kegeles and Heckly (1946) were not able to absorb protective antibody with oedema fluid produced by virulent strains. If such an antigen exists, therefore, it must be so labile that it is destroyed in the extraction process. Another possibility is that, since it is not possible to remove the culture filtrate antigen after absorption, failure to obtain absorption might be due to the formation of a soluble antigen-antibody complex easily dissociated in the animal body. Evidence for a soluble antigen-antibody complex between oedema fluid and the pseudoglobulin fraction of immune serum has been put forward by Grabar and Staub (1944). It is, then, still required to explain why living organisms are necessary to produce circulating antibody against the filtrate antigen. Obviously, considerably more work must be done before these problems can be solved.

Further work on the nature of the protective antigen is also being carried out.

SUMMARY.

1. An antigen having similar properties to that present in anthrax oedema fluid capable of protecting rabbits, sheep and monkeys against 100 or more lethal doses of spores has been produced in filtrates of cultures of *B. anthracis* in plasma or serum of various animals.

2. The size of culture inoculum, the age and pH of the culture are important factors. Destruction of the antigen by the growing organism may occur. No antigen is produced at pH 6·5 or less.

3. Plasma heated to 56° C. for 1/2 hour is still capable of producing the antigen, but not when heated to 70° C.

4. Two factors in plasma or serum are necessary. One is dialysable and can be replaced by sodium bicarbonate. The other is retained and is associated with the serum proteins.

5. The production of the antigen has no relation to virulence, capsulation or sporulation. No virulent strain failed to produce antigen. The production of antigen by avirulent strains was related to their efficacy as living vaccines. No antigen was produced by *B. cereus*.

6. Sera from rabbits and sheep hyperimmunized with the antigen contained no protective antibodies. Further immunization with living virulent bacilli produced effective protective sera. The protective antibodies were not absorbed with the antigen from culture filtrates or with living bacilli.

7. No precipitation or complement-fixation reactions were obtained with hyperimmune sera.

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